Academic Sciences International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 3, Issue 4, 2011

Research Article

IN VITRO ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES OF GRAPES (*VITIS VINIFERA. L*) SEED AND SKIN EXTRACTS - MUSCAT VARIETY

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Received: 30 Jun 2011, Revised and Accepted: 27 July 2011

ABSTRACT

The skins and seeds of grapes are known to be rich sources of phenolic compounds, both flavonoids and non-flavonoids. The aim of the present study is to determine polyphenolic composition, antioxidant potential along with free radical scavenging activity and antimicrobial properties of ethanol extract of Grape skin and seed. Grape seed showed high antioxidant and antimicrobial activity compared to grape skin extract which revealed the medicinal properties of grape seed extract. The IC_{50} values of grape skin and seed extract showed that minimum amount can inhibit the toxic radicals with high antioxidant activity. The grape seed extracts also possess high antimicrobial activity than grape skin extracts.

Keywords: *Vitis vinifera*, polyphenols, *in vitro* antioxidant, anti-microbial, grape seed, grape skin

INTRODUCTION

Many diseases are due to the "oxidative stress" that results from an imbalance between formation and neutralization of pro-oxidants. Oxidative stress is initiated by free radicals, causing protein and DNA damage along with lipid peroxidation and these changes contribute to cancer, atherosclerosis, cardiovascular diseases, ageing and inflammatory diseases. Great attention has been paid over the past years to polyphenols and these are naturally present in fruit and vegetables, which are included in our everyday diet. They are efficient free radical scavengers as they can potentially interact with biological systems and play an important role in preventing human diseases[1,2]. So fruits which naturally have phenolic compounds have been extensively studied for their antioxidant activity and antimicrobial activities.

Grape (*Vitis vinifera*) skins and seeds are considered rich sources of poly-phenolic compounds, mainly monomeric catechin and epicatechin, gallic acid, and polymeric and oligomeric procyanidins[3,4]. Their composition and properties have been extensively investigated, with several reports of the presence of large amounts of phenolic compounds having antioxidant activities. The grape seed polyphenols are flavan-3-ol derivatives and only 4% of grape polyphenols exist in grape pulp. In grape skin there is another type of polyphenol, called anthocyanins, which usually have a purple color and amount to ~30% of total polyphenols in grapes. Resveratrol is mainly contained in the skins of grapes[5]. These grape seed and skin extract compounds act as antimutagenic and antiviral agents[6,7]. Other oligomers are procyanidins (or proanthocyanidins). Recent studies have shown that procyanidins in grape seeds possess anti-inflammatory, anti-arthritic, anti-allergic, anti cancer activities, and it prevents heart disease and skin aging, inhibits carrageenin- or dextran-induced hind paw edema, stabilizes the capillary wall, improves visual performance in humans and also it has shown many other beneficial health effects[8-11].

The aim of this study was to assess the phenolic compounds content and the *in vitro* antioxidant and antimicrobial activities of seed and skin extracts of purple black muscat grape variety from Coimbatore region, with a view to exploiting its potential as a source of natural antioxidants.

MATERIALS AND METHODS

Sample collection

This study was carried out with *Vitis vinifera* L. Muscat from the Coimbatore district of Tamil Nadu, India. Grapes were harvested at their technological maturity, from Thondamoothur region, Coimbatore. Approximately 500 undamaged and disease-free berries were randomly snipped from clusters. The grapes were dried with filter paper and weighed. The skins and seeds were carefully separated manually from pulp, dried with filter paper, weighed and made into powder and stored until analysed.

Extraction

After the grape seeds and skins are shade dried and powdered, they were extracted successively with ethanol in a soxhlet extractor for 18-20 hrs. The extracts were concentrated to dryness under reduced pressure and controlled temperature (40- 50ºC) in a rotavapor. Then the extract was subjected to phytochemical analysis and checked for antioxidant and antimicrobial activities.

Phytochemical analysis of grape seed and skin extracts

Qualitative analysis of phytochemicals[12-14]

The ethanol extracts obtained were subjected to preliminary phytochemical screening and the following tests were done to check the presence of phytoconstituents. Test for Alkaloids (Mayer's test), Flavonoids (Alkaline reagent test), Carbohydrates (Molischs test), Glycosides (Legals test), Saponins, Tannins, Phytosterol (Salkowski test), Triterpenoid (Libermann Burchard test), Proteins and Amino acids (Ninhydrin test), Biuret test, Anthraquinones, steroids, Catachol, Reducing sugars (Fehling's Test), Acidic compounds, Lipids/Fats, Phlobatannins and Resins.

Quantitative analysis of phytochemicals

The total phenol content was determined using Folin-ciocalteau reagent[15] and the total flavonoid content was estimated using aluminium chloride method [16]. Estimation of Ascorbic acid was done according to the method of sadasivam *et al*, 1987 [17].

In Vitro Antioxidant Activity

DPPH scavenging assay[18, 19]

The stable 1,1-diphenyl-2-picryl hydrazyl radical (DPPH) was used for determination of free radical-scavenging activity of the ethanol extracts. Different concentration of substrate, 1.0 ml of (0.1mM) DPPH in ethanol, 550 µl of 50 mM Tris- HCl buffer (pH 7.4) were added and the mixture was incubated for 20 min at room temperature. Absorbance of the mixture was measured using spectrophotometer at 517 nm after 20 mins. Mixture without substrate served as control. Lower absorbance of the reaction mixture indicates higher free radical-scavenging activity. All determinations were performed in triplicate and calculated using the following equation:

Effect of scavenging (%) = [1-A sample (517nm) /A control (517nm)] ×100

Hydrogen peroxide scavenging assay [20]

Hydrogen peroxide solution (2 mM/L) was prepared with standard phosphate buffer (pH 7.4). Different concentration of the extracts in distilled water was added to 0.6 ml of hydrogen peroxide solution. Absorbance was determined at 230 nm after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage inhibition of different concentrations of the extracts was determined and compared with the standard, ascorbic acid.

In all above the method percentage inhibition was calculated by using formula

% Radical scavenged = (Control OD – Test OD) / Control OD × 100

Assay of Reducing Power [21,22]

In test tubes, different concentrations of plant extract solution (100-500 µg/ml) was mixed with 2.5 ml phosphate buffer (0.2 M, pH 6.6) and 2.5 ml potassium ferricyanide (1%, w/v), then mixture was incubated at 50º C for 20 minutes. After incubation, 2.5 ml of trichloroacetic acid (100g/l) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. Finally, 2.5 ml of the supernatant solution was mixed with 2.5 ml of distilled water and 0.5 ml FeCl3 (1g/l) and absorbance measured at 700nm in UV-Visible spectrophotometer. Ascorbic acid was used as standard and phosphate buffer used as blank solution. The absorbance of the final reaction mixture of five experiments was expressed as mean ± standard deviation. Increased absorbance of the reaction mixture indicates stronger reducing power.

Total Antioxidant Capacity

The total antioxidant capacity assay of grape seed and skin extracts was followed by the method of Preito et al, 1999[23]; 1 ml of extract of different concentrations was treated with 1 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) in eppendorf tube. The tubes were capped and incubated in a thermal block at 95 °C for 90 min. After cooling to room temperature, the absorbance of the aqueous solution of each was measured at 695 nm against a blank. Ascorbic acid was used as the standard and the total antioxidant capacity is expressed as equivalents of ascorbic acid.

Determination of antibacterial activity

The antibacterial activity was tested using agar well diffusion and broth dilution methods according to [24,25]. The MTCC cultures were obtained from Kovai Medical Centre Hospitals, Coimbatore, Tamil Nadu. The grape and skin extracts were tested against *Staphylococcus aureus, Klesiella pneumoniae, Enterococcus faecalis, Escherichia coli* and *Pseudomonas aeroginosa.*

Agar well diffusion method

Briefly, 1 ml of the test culture (10^7 CFU/ml) was inoculated into a sterile plate with 20 ml Muller Hinton molten agar and the plate was shaken for even spread and proper mixing of the organisms and agar. It was then allowed to solidify. 5 wells of approximately 6mm in diameter were made on the surface of the agar plates using a sterile borer. The plates were then turned upside down and the wells were labeled with a marker. Stock solution of each plant extract was prepared at concentration of 50 mg/ml in ethanol. Each well was filled with 0.10 ml of the plant extracts. 0.10 ml of ethanol was taken as negative control and 10 mcg of streptomycin served as a positive control respectively. The plates were then incubated at 37°C for 24 hrs and zone of inhibition was measured. The results were then tabulated [26].

Determination of Minimum inhibitory concentration (MIC)

The MIC method was applied on extracts that proved their high efficacy against microorganisms by the disc diffusion method. A stock solution of grape skin and seed extract was prepared in 10% dimethylsulfoxide (DMSO) and then serial dilutions of extracts were made in a concentration range from 10 to 250µg/ml. The 96-well plates were prepared by dispensing, into each well, 95 µl of Mueller Hinton Broth, 100 µl of plant extract and 5 µl of the inoculants. All tubes were incubated at 37 °C for 24hrs. The lowest concentration that did not permit any visible growth when compared with the control was considered as the minimum inhibitory concentration[27].

GC-MS Analysis

The GCMS (Gas Chromatography Mass Spectroscopy) analysis of the Grape seed and skin ethanolic extract was carried out at the South India Textile Research Association (SITRA), Coimbatore. The Gas Chromatography (GC) was carried out by using Thermo GC Trace Ultra Version 5.0 equipment with run time of 35:31 mins and the Mass Spectrometry (MS) was carried out by using Thermo MS DSQ II equipment.

Statistical analysis

All the grouped data were statistically evaluated with SPSS/10 software. Hypothesis testing methods included one way analysis of variance (ANOVA) followed by least significant difference (LSD) test. *P* values of less than 0.05 were considered to indicate statistical significance. All the results were expressed as mean ± S.D. for three experiments in each.

RESULTS AND DISCUSSION

The present study for *in vitro* antioxidant and antimicrobial activity was carried out on *Vitis vinifera* (Muscat variety) seed and skin extracts. These revealed the presence of phytochemicals with biological activity that can be of valuable medicinal value. For example, Phytochemicals such as saponins, terpenoids, flavonoids, tannins, steroids and alkaloids have anti-inflammatory effects[28-30]. Glycosides, flavonoids, tannins and alkaloids have hypoglycemic activities[31,32]. Steroids and triterpenoids showed the analgesic properties [33]. The phytochemical screening of grape seed and skin showed the presence of alkaloids, flavonoids, carbohydrates, saponins, tannins, triterpenoids, catechol, steroids, phlobatannins and acidic compounds. Resins and lipids were present in grape seed alone. Glycosides, phytosterol were absent in both grape seed and skin extracts (Table 1).

Table 1: Phytochemical constituents of Grape seed and skin extracts

Phytochemicals	Grape seed	Grape skin
Alkaloids	+	+
Flavonoids	+	+
Carbohydrates	+	+
Glycosides	_	_
Saponins	+	+
Tannins	+	+
Proteins	+	+
Amino acids	+	+
Phytosterol	_	_
Triterpenoids	+	+
Phlobatannins	+	+
Lipids/ Fats	+	_
Reducing Sugar	+	+
Steroids	+	+
Resins	+	_
Acidic compounds	_	_
Catechol	+	+

+ - Presence, -- - Absence

Quantitative estimation showed high amount of phenols, flavonoids and ascorbic acid (Table 2).

Table 2: Determination of total phenols, total carbohydrates, total ascorbic acid and total flavonoids content in Grape seed and skin extracts

Extract	Phenol (mg/g)	Carbohydrates (mg/g)	Ascorbic acid (mg/g)	Flavonoids (mg/g)
Grape seed	196±15.18	180±14.23	0.34±0.22	56±4.12
Grape skin	150±10.11	160±9.19	0.11±0.09	34±3.23

Grape seed showed high amount of phenol and flavonoids when compared with grape skin extracts.

***In vitro* antioxidant activity**

The antioxidant activity of grape seed and skin extracts were evaluated using various antioxidant assays like DPPH radical scavenging, total antioxidant capacity, Hydrogen peroxide scavenging assay and Reducing power assay. The free radical scavenging activity of grape seed and skin extracts were determined by the DPPH method and the results are shown in Figure 1.

Antioxidant molecules can quench DPPH free radicals and convert them to a colourless product, resulting in a decrease in absorbance at 517 nm [34]. In our study, the grape seed extracts exhibited appreciable scavenging activity when compared with grape skin extracts. The IC_{50} values of grape skin extract was 15 µg/ml and for grape seed extract was 11 µg/ml when compared to ascorbic acid having 5 µg/ml. Total antioxidant capacity of grape seed and skin extracts is expressed as the number of equivalents of ascorbic acid. The phosphomolebdnum method was based on the reduction of Mo (VI) to Mo (V) by the antioxidant compound and the formation of a green phosphate/Mo (V) complex with a maximal absorption at 695 nm. The study revealed that the antioxidant activity of the grape seed extract exhibits increased percentage of inhibition when compared with grape skin extracts (Figure 2).

The IC_{50} values of grape skin extract was 9 µg/ml and for grape seed extract was 7 µg/ml when compared to ascorbic acid having 2 µg/ml. The reducing power property indicates that the antioxidant compounds are electron donors and can reduce the oxidized intermediates of the lipid peroxidation process[35]. The reducing power of grape seed and skin extracts increases with increasing concentration (Figure 3).

The measurement of H_2O_2 scavenging activity is one of the useful methods of determining the ability of antioxidants to decrease the level of pro-oxidants such as H_2O_2. Hydrogen peroxide itself is not very reactive, but sometimes it can be toxic to cells because of rise in the hydroxyl radicals in the cells[36, 37]. The hydrogen peroxide scavenging showed higher inhibition for higher concentrations of the extract. Grape skin extract showed high activity than grape seed extract (Figure 4).

The free radical scavenging and antioxidant activities may be attributed to the presence of phenolic compound and flavonoid[38].

Antimicrobial activity- Agar well diffusion method and MIC:

Microbial activity is a primary cause of the deterioration of many foods and is often responsible for the loss of quality and safety. The results of the antimicrobial activity by the agar well diffusion method of grape seed and skin extracts were presented in Table 3.

Both the extracts showed good bactericidal activity against the Gram negative and Gram positive bacteria. Grape seed extract showed high antibacterial activity (Figure 6) when compared with grape skin extract (Figure 5) against all the five organisms.

Streptomycin was used as a positive control and ethanol as negative control. The zone of inhibition showed the range of 8-16 mm for grape seed extracts and 3-8 mm for grape skin extracts. The discovery of a potent remedy from plant origin will be a great advancement in bacterial infection. The result of present investigation highlights that the antibacterial potentiality of the extracts of grape seed and skin extracts.

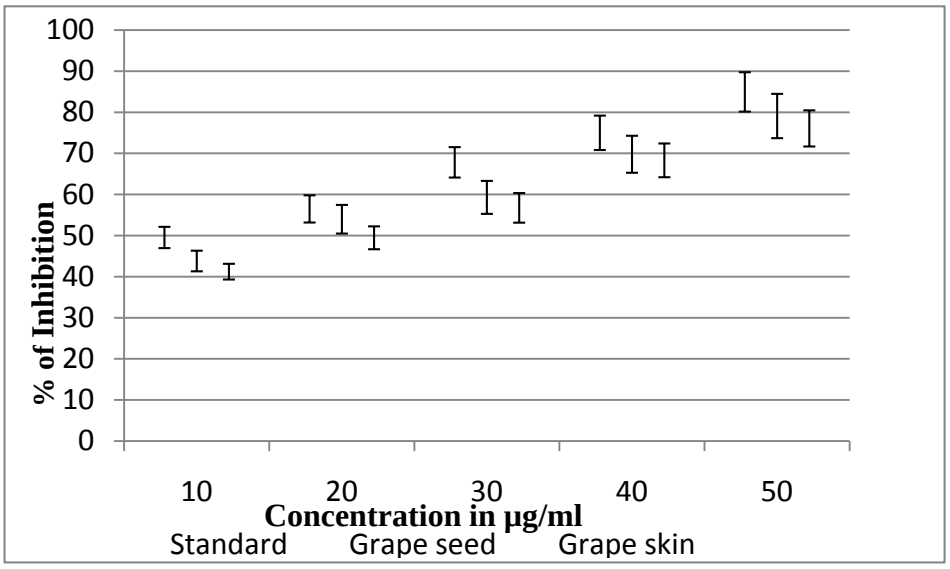


Fig. 1: Scavenging activity of grape skin and seed extracts on DPPH radical

Values are mean ± SD of three determinations.

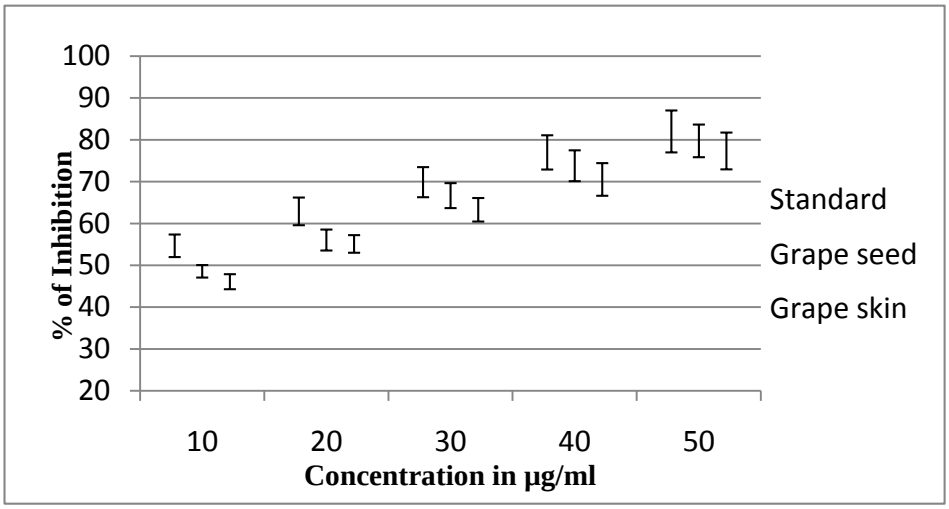


Fig. 2: Total Antioxidant activity grape skin and seed extracts

Values are mean ± SD of three determinations.

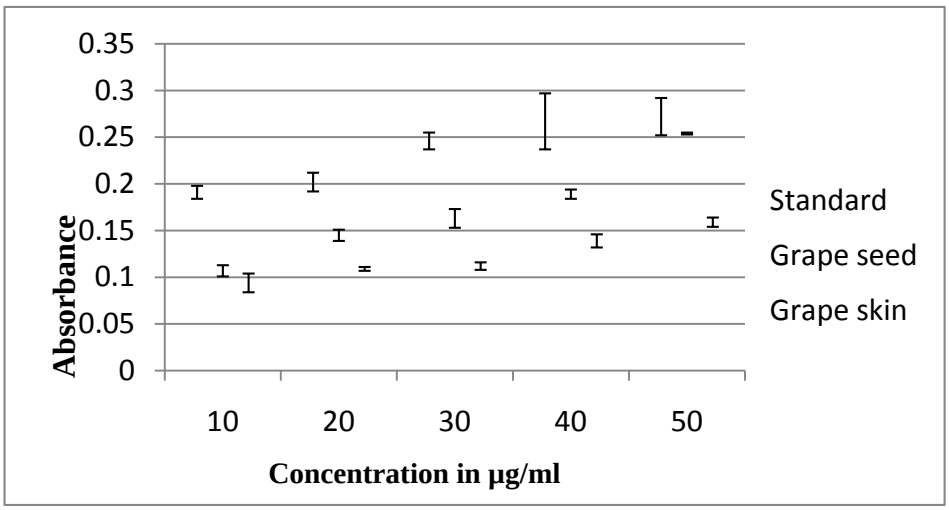


Fig. 3: Reducing power assay of grape skin and seed extracts

Values are mean ± SD of three determinations.

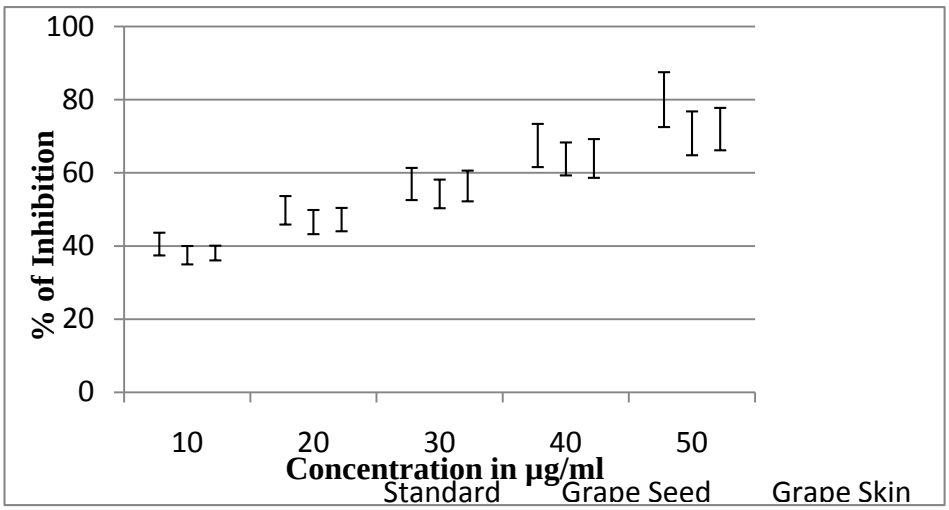


Fig. 4: Effect of grape skin and seed extracts on the scavenging of H_2O_2

Values are mean ± SD of three determinations.

The IC_{50} values of grape skin extract was 15.1 µg/ml and for grape seed extract was 16 µg/ml when compared to ascorbic acid having 14 µg/ml.

Table 3: Antibacterial Activity of Grape seed and skin extracts

Plant Extract (mg/ml)		Zone of Inhibition (mm)				
		E.coli	*K.pneumoniae*	*E. faecalis*	*S.aureus*	*P.aeroginosa*
Grape Seed	50	8.3±0.15	8.3±0.11	8.3±0.1	10±0.1	9.9±0.05
	100	10±0.1	10±0.5	12±0.15	12.1±0.1	12.1±0.05
	150	12±0.15	12.2±0.05	13±0.2	13±0.1	12.2±0.05
	200	14.2±0.2	14.1±0.1	14±0.1	14.2±0.2	13±0.1
	250	15 ±0.1	16±0.1	16.2±0.1	16.2±0.05	14.2±0.2
Grape Skin	50	3±0.15	3±0.5	3±0.1	3.1±0.15	2.3±0.1
	100	4.3±0.1	4.2±0.05	4.3±0.11	4±0.1	3.2±0.2
	150	5±0.15	6.3±0.15	3.9±0.25	6.2±0.2	4±0.17
	200	6±0.1	7.2±0.25	5.1±0.1	6.1±0.1	5.2±0.2
	250	7±0.5	8.3±0.1	5.9±0.05	7±0.5	6±0.1
Streptomycin		16.2±0.2	18±0.1	6±0.17	18±0.2	16±0.15
Control		2.3±0.1	2.3±0.11	2.2±0.15	2±0.15	2.2±0.2

Data are expressed as mean ±S.D.

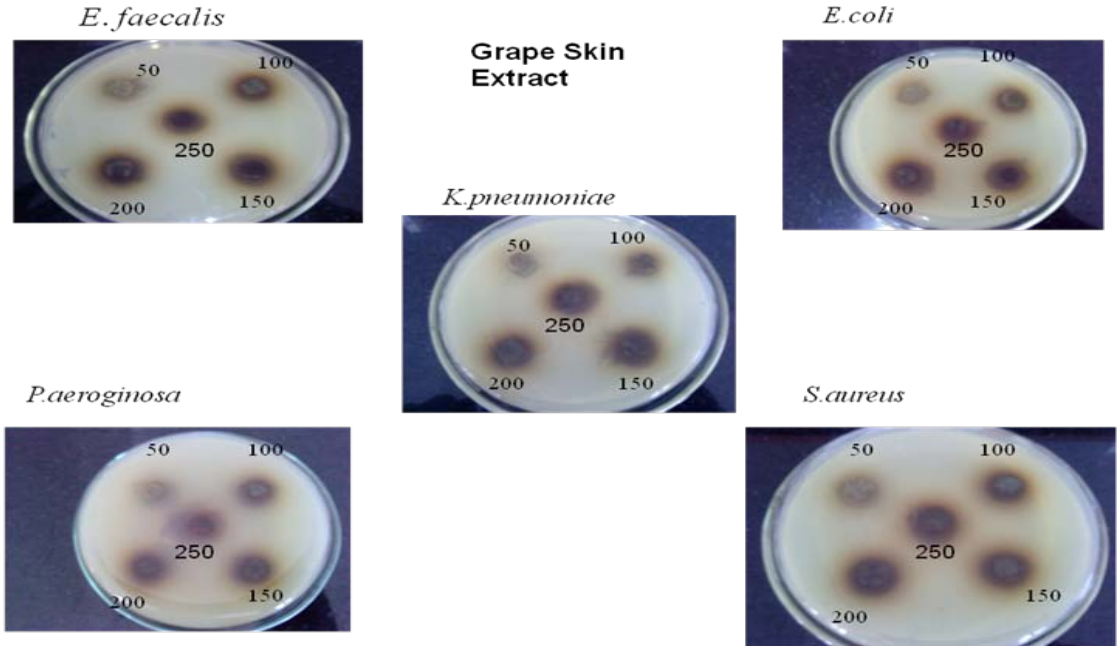


Fig. 5: Photographs showing the agar well diffusion method of Grape skin extract

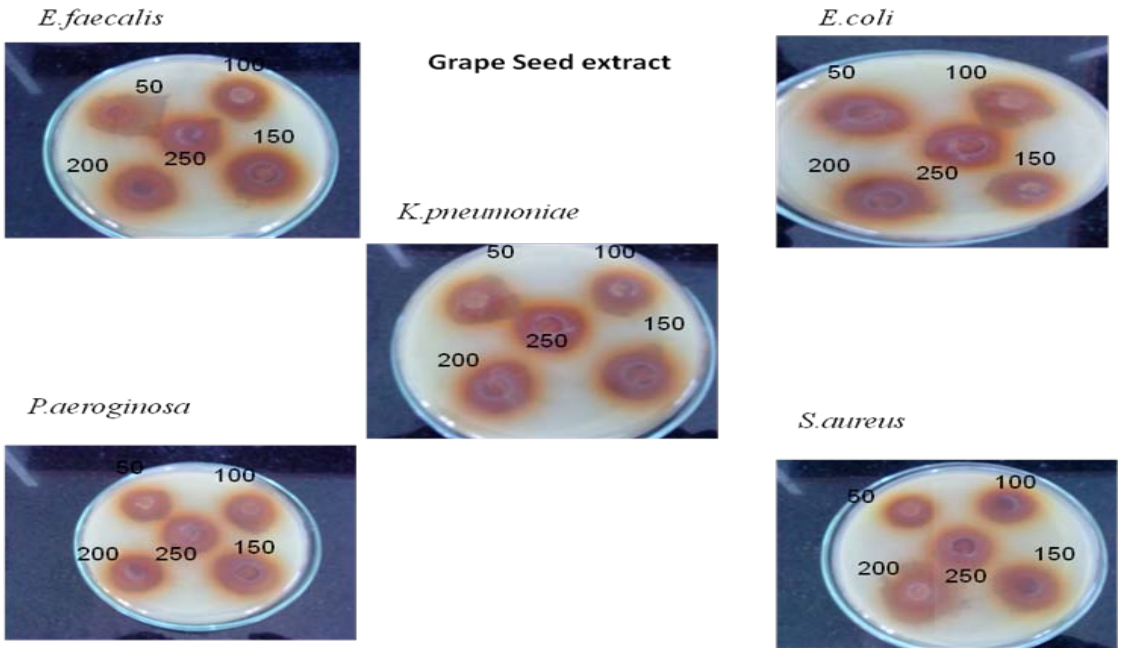


Fig. 6: Photographs showing the agar well diffusion method of Grape seed extract

The minimum inhibitory concentration showed that grape seed extract inhibits in minimum concentration of 30µg/ml, and grape skin extracts inhibits at a concentration of 40µg/ml (Table 4).

Table 4: Determination of Minimum Inhibitory Concentration

Concentration of plant extract (µg/mL)		Bacterial Species				
		E.coli	*K.pneumoniae*	*E. faecalis*	*S.aureus*	*P.aeroginosa*
Grape Seed	10	–	–	–	–	–
	20	–	–	–	–	–
	30	+	–	–	–	+
	40	+	+	–	+	+
	50	+	+	+	+	+
	60	+	+	+	+	+
Grape Skin	10	–	–	–	–	–
	20	–	–	–	–	–
	30	–	–	–	–	–
	40	+	–	+	+	+
	50	+	+	+	+	+
	60	+	+	+	+	+

- Indicates 'growth', + indicates 'no growth'

The mode of action may be due to surface interaction of sterol molecules present in the extracts with the bacterial cell wall and membrane leading to alteration in the primary structure of cell wall and membrane, ultimately leading to pore formation and degradation of the bacterial components[39]. Finally, it can be concluded that the Muscat variety of black grapes are rich in antioxidants and possess antimicrobial activity which can be useful for pharmaceutical or food industry. These high activities are due to the high amount of phenols present in the grape seed and skin. Generally, we separate the seeds from the skin and pulp of grapes before eating which should be avoided as it possess high antioxidant activity than the other parts.

GC-MS Analysis

In the GCMS analysis, nearly 7 compounds were identified in the *Vitis vinifera* seed and skin extracts. The identification of the phytoconstituents was based on the peak area, retention time and molecular weight. The major compounds identified were tabulated below (Table 5 and 6).

Table 5: GCMS analytical report for major phytoconstituents present in ethanolic extract of *Vitis vinifera* skin extract

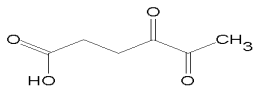
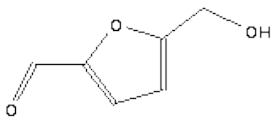
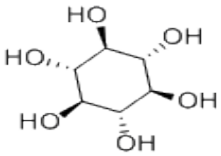
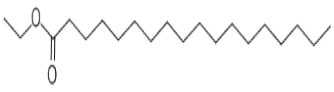
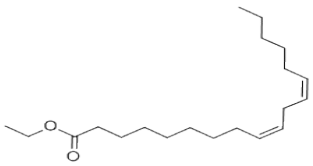
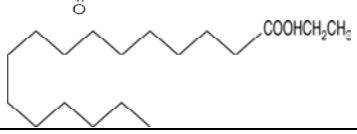
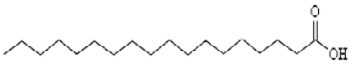
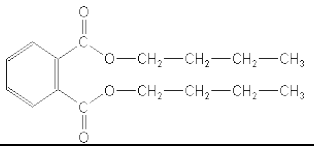
RT	Name of the compound	Molecular Formula	Molecular weight	Peak area (%)	Structure
8.06	4,5-Dioxohexanoic acid	$C_6H_8O_4$	144	4.11	
9.70	2-Furancarboxaldehyde, 5-(hydroxymethyl)- (CAS)	$C_6H_6O_3$	126	18.10	
16.71	Quercinitol	$C_6H_{12}O_6$	180	4.76	
22.66	ethyl stearate	$C_{20}H_{40}O_2$	312	11.92	
25.64	Ethyl linoleate	$C_{20}H_{36}O_2$	308	8.70	
32.56	Hexadecanoic acid, ethyl ester (CAS)	$C_{18}H_{36}O_2$	284	2.36	

Table 6: GCMS analytical report for major phytoconstituents present in ethanolic extract of *Vitis vinifera* seed extract

RT	Name of the compound	Molecular Formula	Molecular weight	Peak area (%)	Structure
13.44	Stearic acid	$C_{18}H_{36}O_2$	284	0.24	
22.37	Phthalic acid	$C_{16}H_{22}O_4$	278	37.43	

The GCMS chromatogram of *Vitis vinifera* seed and skin extracts are shown in Figure 7 and 8.

Gas Chromatography Mass Spectroscopy (GCMS) analysis

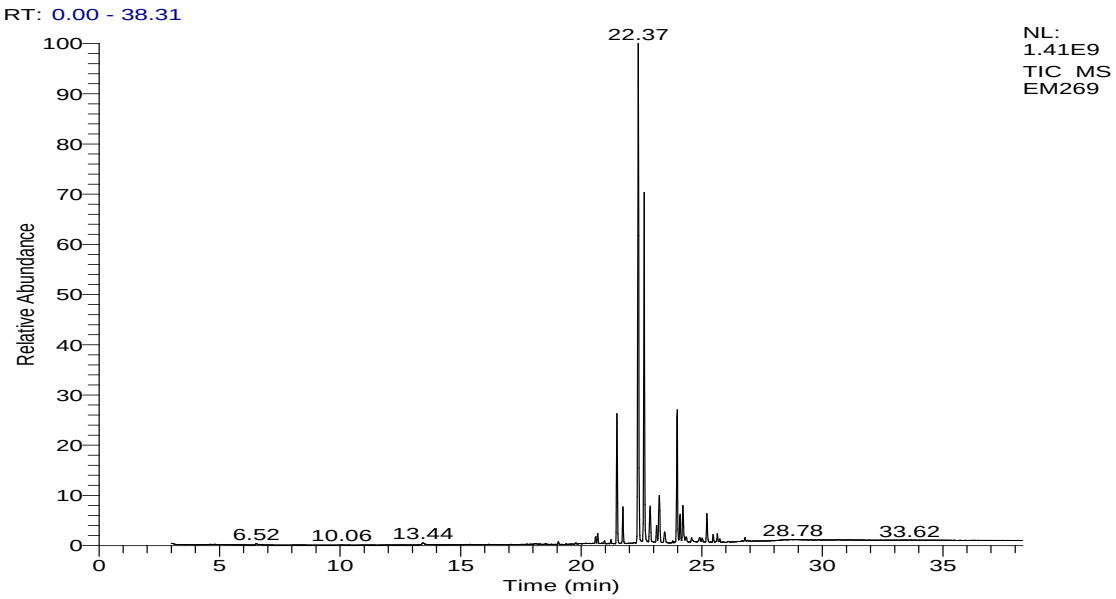


Fig. 7: The GCMS chromatogram of the *Vitis vinifera* seed ethanolic extract

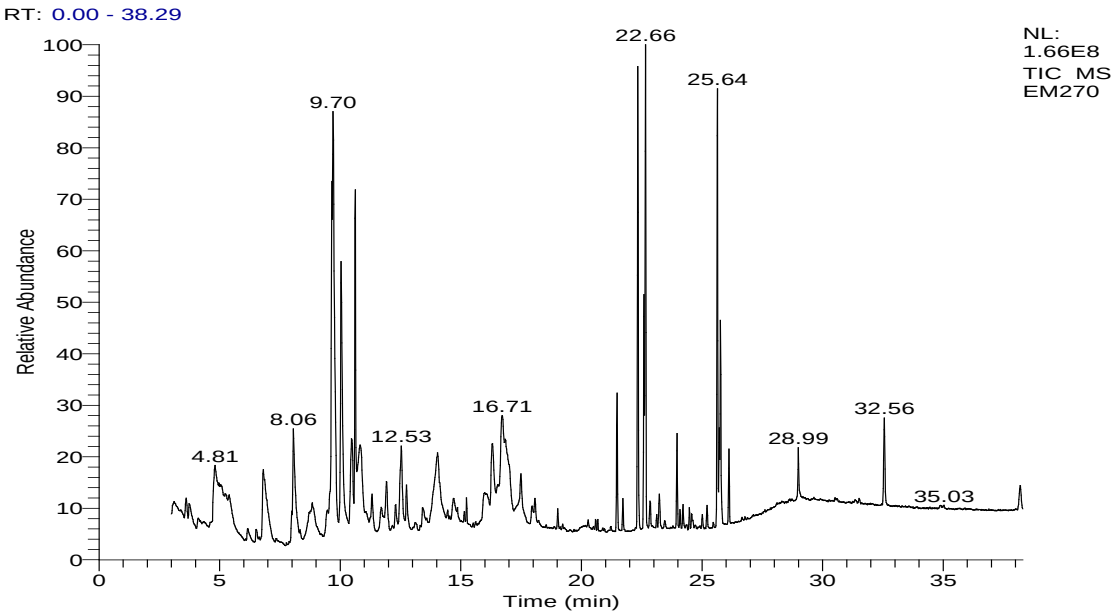


Fig. 8: The GCMS chromatogram of the *Vitis vinifera* skin ethanolic extract

ACKNOWLEDGEMENT

We would like to express our appreciation to Dr. Paul Dinakaran, Chancellor, Dr. Paul. P. Appasamy, Vice-Chancellor and Dr. Anne Mary Fernandez, Registrar, Dr. Patrick Gomez, Director, School of Biotechnology and Health Sciences, Karunya University, Coimbatore for providing necessary facilities to carry out the experiments.

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